

Improvement of Mouse β -Thalassemia by Recombinant Human Erythropoietin

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Homozygous β thalassemic mice received 50 U (1,660 U/kg) of recombinant human erythropoietin (rhEpo) 5 days a week for 2 weeks. Hemoglobin increased from 9.2 ± 0.6 g/dL to 10.5 ± 0.4 g/dL ($P = .002$) and hematocrit increased from $29.2\% \pm 0.9\%$ to $34.1\% \pm 1.9\%$ ($P = .0014$). The β minor/ α globin chain synthesis ratio increased slightly but significantly between day -4 (0.75 ± 0.07) and day 4 (0.81 ± 0.04) ($P = .01$) and reached a minimum ratio (0.67 ± 0.03) on day 15 ($P = .001$), being parallel to reticulocyte counts and to the incorporated trichloroacetic acid (TCA)-insoluble radioactivity, therefore parallel to the erythropoietic output in thalassemic mice, as in normal mice. Erythrocyte defects were

improved in β thalassemic mice treated by rhEpo: membrane-associated α globin was significantly decreased ($P < .01$), thiol group reactivity of ankyrin was significantly improved ($P < .05$), spectrin alterations were reduced, and deformability of mouse thalassemic red blood cells was normalized. These results provide experimental criteria for modulating globin chain imbalance necessary for the therapy of human β thalassemia intermedia, and suggest that rhEpo might be of interest to improve the red blood cell mass and reduce erythrocyte alterations in this disease.

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DESPITE A REMARKABLE understanding of the molecular defects responsible for human β thalassemia, there is still no safe and specific therapy for Cooley's anemia. The availability of a good animal model for this pathology should be helpful in devising new therapeutic approaches, especially in increasing fetal hemoglobin (Hb F) synthesis and reducing globin chain imbalance.

Murine β thalassemia occurred spontaneously in a DBA/2J mouse¹ as a consequence of the entire deletion of β major globin gene.² Homozygous β thalassemic mice have clinical and biologic features similar to those observed in human β thalassemia intermedia.³ The murine β thalassemia was shown to be a good model for the erythrocyte defects of the human disease³: increase in insoluble α chains, and decrease in spectrin and in the thiol group reactivity of spectrin and ankyrin. In the DBA/2J strain in which β thalassemia was found, normal adult Hb phenotype consists of 80% major Hb and 20% minor Hb, as the result of the relative expression of the two β globin genes, β major and β minor. In homozygous β thalassemic mice, the absence of β major globin chain is partially corrected by an increase in β minor chain synthesis, resulting in a β/α globin chain synthesis ratio of 0.7 to 0.8. This relative increase in β minor chain synthesis in β thalassemia in comparison with that observed in the normal state has been shown to occur at the translational level.⁴

No Hb equivalent to human HbF has been unequivocally demonstrated in the mouse. A recent study reported that the murine $\epsilon\gamma$ gene was expressed later than the other embryonic gene $\beta\eta$.⁵ However, this $\epsilon\gamma$ gene is already expressed during the embryonic stage of development in

yolk sac blood islands; the switch from $\epsilon\gamma$ to mouse adult β globins does occur at the middle of gestation, much earlier than in the human. Several studies indicated that the β minor globin chain synthesis has some features of HbF synthesis in humans. The β minor globin chain synthesis has been reported to decline during ontogeny,^{6,7} and to increase under conditions that are associated with enhanced HbF synthesis in humans: erythropoietic stress,⁸ congenital macrocytic anemias,⁹ and thalassemia.^{4,10} Moreover, β minor globin chain synthesis has been shown to increase in response to cytotoxic agents such as 5-azacytidine,¹¹ as does γ globin chain synthesis in human.¹²

In humans, recombinant human erythropoietin (rhEpo) is now widely used to reduce anemia due to chronic renal failure. In mice, rhEpo has been proved to be effective in a congenital anemia due to a stem cell defect¹³ and in some types of hemolytic anemia.¹⁴ The fact that Epo not only stimulates erythropoiesis quantitatively, but also increases the proportion of HbF synthesis in baboons,¹⁵ as well as β minor globin synthesis in normal mice,¹⁶ prompted us to evaluate the potential benefit of rhEpo injections to homozygous β thalassemic mice.

We show in this report that rhEpo increased Hb and hematocrit (Hct), changed β minor globin chain synthesis, and improved the erythrocyte abnormalities observed in homozygous β thalassemic mice.

MATERIALS AND METHODS

Animals

Normal DBA/2J mice (Hb haplotype: Hbb⁺/Hbb⁺) were obtained from IFFA CREDO (Saint-Germain-sur-l'Arbresle, France). Mice homozygous for β thalassemia (Hb haplotype: Hbb⁰/Hbb⁰) were generously provided by F. Costantini (Columbia University, NY). Mice used in this study were 12 months old.

rhEpo Injections

Six normal mice and six homozygous β thalassemic mice received 50 U of rhEpo (Amersham, Bucks, UK) (1,660 U/kg) diluted in sterile saline buffer containing 10 mg/mL bovine serum albumin, administered 5 days a week for 2 weeks. rhEpo was injected intraperitoneally. The first day on which mice received rhEpo is referred to as day 0. Mice were bled 80 μ L 4 days before the first rhEpo injection (day -4) and 4 days after the last injection (day 15). They were bled 40 μ L on days 4 and 8. Two normal mice (nos. 5 and 6) received iron (Fer injectable Lucien), 2 mg/kg, on days 4, 8, and 11 in addition to rhEpo. Blood was collected in heparinized

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capillary tubes. Mice were killed simultaneously on day 15 with control normal and thalassemic mice to allow study of membrane proteins and detection of anti-rhEpo antibodies.

Hematology

Hb concentration was determined by the spectroscopic measurement of the cyanmet derivative. Hct was determined by centrifugation in a micro-Hct centrifuge. Reticulocytes were counted after staining with brilliant cresyl blue.

Globin Chain Synthesis

Red blood cells (RBCs) were washed twice with cold saline buffer (NaCl 0.15 mol/L) and once with NCTC 109 medium (Sigma Chemical Co. St Louis, MO) supplemented with glutamine 1 mmol/L and devoid of leucine. Twenty microliters of RBCs, suspended in NCTC 109 medium (adjusted to an Hb value of 10 g/dL), were added to 10 μ Ci of 3 H-leucine (CEA, Saclay, France) present in 10 μ L of medium. The incubation was allowed to proceed for 30 minutes at 37°C in a shaking water bath. The incubation was stopped by cooling the samples in ice. Two microliters were withdrawn to measure radioactivity incorporated into the trichloroacetic acid (TCA)-insoluble material. The cells were washed three times with cold saline solution and stored in liquid nitrogen until analyzed.

Globin chain composition and synthesis ratio were determined by electrophoresis of 50 μ g protein on polyacrylamide gels containing Triton, acetic acid, and urea (urea-Triton-polyacrylamide gel electrophoresis [UT-PAGE]).¹¹ Coomassie blue-stained gels were scanned at 570 nm on a Preference densitometer (SEBIA, Issy les Moulineaux, France) and then submitted to fluorography.¹⁴ Kodak X Omat-AR films (Kodak) were scanned when a globin chain had a fixed integrated optical density value. The proportion of β minor globin chain synthesis was determined in comparison with α chain synthesis (β minor/ α ratio), or to β chain synthesis (β minor/ β minor + β major ratio).

Study of the Membrane Skeletal Proteins and of Their Reactive Thiol Groups

Alterations in membrane proteins were examined by UT-PAGE of cell ghosts¹⁵ prepared according to the method of Dodge et al¹⁶ in the presence of 0.1 mmol/L phenylmethylsulfonyl fluoride to inhibit proteolysis. Labeling of the membrane ghosts with 3 H-N-ethyl-maleimide (3 H-NEM) was performed as previously described¹¹ to determine by fluorography the distribution of the most reactive thiol groups of membrane proteins. Protein and reactive thiol group distribution were expressed as the percentage of the total membrane proteins (membrane specific and skeletal proteins) excluding globin. Membrane-associated globin chains were expressed as the percentage of membrane proteins (ie, excluding globin).

Rheologic Properties of the Cells

The deformability of normal and thalassemic erythrocytes was assessed with a cell transit analyzer (CTA; ABX, Levallois, France) that measures the time required for each erythrocyte to pass through a membrane pore (Oligopore) of 5.0 μ m in diameter and 15.0 μ m in length. The same filter was used throughout the study. The individual transit times, in milliseconds (ms), of 2,000 cells under a driving pressure of 3 cm of H₂O were determined for each sample. The mean cell transit time (Tm), and the times required for the 25, 50, 75, 90, and 95 percentile of cells to pass through the micropore filter were automatically determined by the CTA.

Measurement of Endogenous Immunoreactive Epo in Control Normal and Homozygous β Thalassemic Mice

Three normal DBA/2J and four homozygous β thalassemic mice (12 months old) were used to determine endogenous serum Epo levels. Epo levels could not be determined in mice that received rhEpo injections, but could be determined in control β thalassemic mice having the same age because of the large amount of serum that is required, and because bleeding of the mice could increase endogenous Epo production and interfere with the effects of rhEpo injections. Serum Epo levels were measured by a radioimmunoassay using 125 I-rhEpo (Amersham) as a tracer and a polyclonal anti-Epo antibody raised in rabbits.

Detection of Antibodies to rhEpo

Normal and thalassemic mice that had been treated with rhEpo were bled 5 days after the last rhEpo injection to detect antihuman Epo antibodies. Detection of antibodies to rhEpo was performed by radioimmunoprecipitation. Mice serum was diluted to 1/10. Antigen-antibody complexes were precipitated either with *Staphylococcus* Protein A or with 6% polyethylene glycol 6000 (PEG).

Statistical Analysis

Data are shown as mean \pm 1 standard deviation (SD) and were analyzed with Student's *t*-test.

RESULTS

Hematology

Normal mice. In normal mice, 10 rhEpo injections (50 U/injection) induced a dramatic increase of Hb concentration and Hct (Table 1); Hb increased from 13.5 ± 0.2 g/dL to 19.2 ± 0.4 g/dL ($P = .0001$), and mean Hct from $44.6\% \pm 0.9\%$ to $61.6\% \pm 2.0\%$ ($P = .0001$). Hb concentration increased very rapidly during the first days of rhEpo injections, and more slowly afterwards. Reticulocyte counts were $1.0\% \pm 0.5\%$ in the basal state, $12.5\% \pm 3.8\%$ on day 4, $3.0\% \pm 0.8\%$ on day 8, and $0.9\% \pm 0.4\%$ on day 15. Two normal mice receiving injectable iron, in addition to rhEpo, did not differ in their hematologic response (data not shown).

TCA-insoluble radioactivity reflects the global capacity of circulating cells to synthesize new proteins, mainly globin chains. It depends, therefore, on the presence of reticulocytes, but also of circulating erythroblasts. Incorporated TCA-insoluble radioactivity was parallel to reticulocyte counts (Table 2); it increased on day 4 ($P = .0001$), similar to the basal state on day 8, and significantly decreased on day 15 ($P = .01$). Analysis of globin chain synthesis (Fig 1) indicated that the proportion of β minor globin chain synthesis was parallel to the incorporation of TCA-insoluble radioactivity. The β minor/ α synthesis ratio increased significantly between day -4 (0.36 ± 0.02) and day 4 (0.41 ± 0.04) ($P = .02$), and reached a minimum ratio on day 15 (0.24 ± 0.04) ($P = .002$). These changes in β minor/ α ratio were not due to modifications in α chain synthesis, because β minor/ β minor + β major synthesis ratio exhibited the same changes (Table 2).

Analysis of nonradioactive globin chain composition by UT-PAGE showed a small increase in β minor globin (from $23.1\% \pm 1.9\%$ on day 0 to $26.5\% \pm 1.7\%$ of total β globin

Table 1. Evolution of Hematologic Parameters During rhEpo Treatment

Day	-4	+4	+8	+15	P Values
Normal mice					
Hb (g/dL)	13.5 ± 0.2*	17.1 ± 0.5	18.1 ± 0.3	19.2 ± 0.4*	.0001
Reticulocytes (%)	1.0 ± 0.5*	12.5 ± 3.8*	3.0 ± 0.8	0.9 ± 0.4	.001
Hct (%)	44.6 ± 0.9*	ND	ND	61.6 ± 2.0*	.0001
β Thalassemic mice					
Hb (g/dL)	9.2 ± 0.5*	10.6 ± 0.6	10.6 ± 0.8	10.5 ± 0.4*	.0001
Reticulocytes (%)	26.4 ± 1.8*	29.3 ± 3.9	16.6 ± 5.2	12.9 ± 2.2*	.001
Hct (%)	29.2 ± 0.9*	ND	ND	34.1 ± 1.9*	.0001

Data shown are mean ± 1 SD.

Abbreviation: ND, not determined.

*Parameters that have been compared together for the statistical P value.

on day 8) that did not reach statistical significance ($P = .07$), possibly because analysis at the nonradioactive protein level is a reflection of the entire population of RBCs (ie, reticulocytes and older cells, including cells that have a life span longer than the duration of the study).

Homozygous β thalassemic mice. In homozygous β thalassemic mice, rhEpo induced a significant increase of Hb and Hct (Table 1). Hb concentration increased from 9.2 ± 0.6 g/dL before the first rhEpo injection to 10.5 ± 0.4 g/dL at the end of treatment ($P = .002$). Hct increased from $29.2\% \pm 0.9\%$ to $34.1\% \pm 1.9\%$ ($P = .001$). The increase in Hb was rapid, reaching a plateau value at day 4. Reticulocyte counts were $26.4\% \pm 1.8\%$ in the basal state, $29.3\% \pm 3.9\%$ on day 4, $16.6\% \pm 5.2\%$ on day 8, and $12.8\% \pm 2.2\%$ on day 15.

In β thalassemic cells, TCA-insoluble radioactivity reflecting protein synthesis was parallel to reticulocyte counts, ie, slightly increased on day 4, and significantly decreased on days 8 and 15 (Table 2). Similarly, the β minor/α globin chain synthesis ratio increased significantly between day -4 (0.75 ± 0.07) and day 4 (0.81 ± 0.04) ($P = .02$), and reached a minimum value (0.67 ± 0.03) ($P = .001$) on day 15, indicating thereby that synthesis of the β minor globin chain is dependent on the erythropoietic stimulation in thalassemic as well as in normal mice.

Endogenous Epo levels were determined in three normal mice and four homozygous β thalassemic mice. The mean endogenous Epo level was 22 ± 12 mU/mL in normal mice, and much higher (182 ± 122 mU/mL) in β thalassemic

mice without statistical significance ($P = .08$) because of the wide interindividual variations in the β thalassemic mice.

Membrane Alterations

Using UT-PAGE of the membrane ghosts after labeling with ³H-NEM and fluorography, we compared the membrane proteins of mice that had been treated with rhEpo with those of control mice (Table 3 and Fig 2). This method evaluates both the proportion of proteins in the erythrocyte ghost and the distribution of their reactive thiol groups that are reporters of the oxidation of membrane proteins.

In comparison with normal mice, untreated β thalassemic mice exhibited a dramatic increase in membrane-bound α globin ($P = .002$), a decrease in spectrin/membrane proteins ratio ($P < .001$), and a diminution in the thiol reactivity of ankyrin ($P = .02$). In addition, protein fractions migrating between band 3 and globin were increased, although not significantly ($P = .17$), and thiol reactivity of spectrin was decreased ($P = .09$). All these abnormalities are similar to those observed in human β thalassemia intermedia.^{1,20}

In normal mice, rhEpo injections induced little changes: spectrin ratio increased slightly but significantly from $28.8\% \pm 0.3\%$ to $29.7\% \pm 0.5\%$ ($P = .02$). The thiol group distribution of normal membrane proteins was not changed by rhEpo injections (Fig 2).

In β thalassemic mice, rhEpo decreased significantly membrane-bound α globin from $17.9\% \pm 3.4\%$ to $7.7\% \pm 4.1\%$ ($P = .008$). Besides, it increased the proportion of

Table 2. Evolution of TCA-Insoluble Radioactivity and β Minor Globin Chain Synthesis During rhEpo Treatment

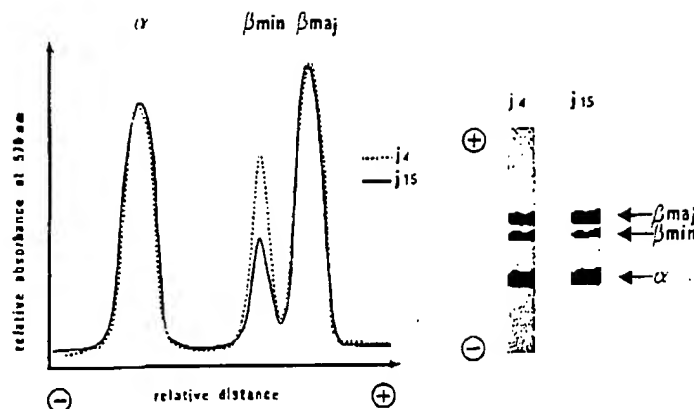
Day	-4	+4	+8	+15	P Values
Normal mice					
TCA ($\times 10^3$ cpm)	52 ± 21*†	192 ± 11*	58 ± 20	22 ± 5†	*.0001 †.01
β min/α synthesis ratio	0.36 ± 0.02*†	0.41 ± 0.04*	0.30 ± 0.05	0.24 ± 0.04†	*.02 †.002
β min/total β synthesis ratio	0.27 ± 0.01*†	0.33 ± 0.02*	0.24 ± 0.03	0.21 ± 0.03†	*.001 †.01
β Thalassemic mice					
TCA ($\times 10^3$ cpm)	193 ± 15*	207 ± 12	137 ± 19	94 ± 42*	*.002
β min/α synthesis ratio	0.75 ± 0.07*	0.81 ± 0.04*†	0.69 ± 0.02	0.67 ± 0.03†	*.015 †.001

β minor/α and β minor/total β synthesis ratio were determined by densitometry of the autoradiographs after UT-PAGE. Data shown are mean ± 1 SD.

Parameters compared together for P value with ().

†Parameters compared together for P value with (†).

Fig 1. Analysis of globin chain synthesis in normal mice. The globin chain synthesis ratio was determined by electrophoresis of 50 μ g of proteins on UT-PAGE, followed by fluorography and scanning of the autoradiographs when a globin chain had a fixed integrated optical density value. Autoradiographs and optical density profiles are from one normal mouse at days 4 and 15 of rhEpo injections.



spectrin, but not significantly ($P = .16$); and decreased the fraction of proteins migrating between band 3 and globin chains ($P = .06$). The thiol group reactivity of ankyrin, which is most impaired in β thalassemic RBCs, was significantly improved ($P = .045$) (Fig 2). Spectrin thiol groups also exhibited an increased reactivity, but this increase did not reach statistical significance ($P = .178$). These data clearly indicate that rhEpo treatment reduced membrane protein alterations of circulating β thalassemic RBCs.

Cell Deformability

Analysis of the deformability of normal mouse RBCs by using a CTA did not show any statistical difference in the Tm or percentiles (percentiles 25, 50, 75, 90, and 95) when comparing the data obtained before (Tm = 1.017 ± 0.041 ms) and after (Tm = 0.972 ± 0.050 ms) rhEpo treatment.

In contrast, the rheologic study of blood cells of untreated thalassemic mice showed that the different parameters (Tm and percentiles 25, 50, 75, 90, and 95) were significantly different from those of normal mice ($P < .05$ to $P < .001$). After rhEpo treatment, the Tm of thalassemic cells was significantly shortened (from 1.155 ± 0.360 to 1.023 ± 0.045 ms; $P = .0009$). The times required for the 25, 50, 75, 90, and 95 percentile of cells to pass through the micropore filter was improved and did not differ any more from the times observed with normal cells (Fig 3). These results clearly show that rhEpo injections not only stimulated erythropoiesis quantitatively in β thalassemic mice, as

assessed by the significant increase of Hb concentration and Hct, but also had a significant effect on the functional properties of circulating thalassemic RBCs.

DISCUSSION

Normal mice receiving 1,660 U/kg rhEpo 5 times a week for 2 weeks had a dramatic increase in their Hct and Hb. Yet, Hb increased very rapidly during the first days of rhEpo injections, and much less rapidly afterwards. Reticulocyte counts were high at day 4 and low on days 8 and 15. These results can be explained by the findings of Alter et al,¹⁴ who injected rhEpo subcutaneously into normal DBA/2J mice, at the same dose and rhythm, and reported that reticulocytes were increased on days 7 and 11 but decreased to less than 2% on day 9 (associated with no rhEpo administration on days 5 and 6). The low reticulocyte counts observed on days 8 and 15 in our experiment probably reflect the temporary and delayed stop of erythropoiesis stimulation that occurred during days 5, 6, and 12 to 15, when no rhEpo was injected and when endogenous Epo production was downregulated because of the polycythemia induced by the treatment.

The reticulocyte counts observed in our study were parallel to that of TCA-insoluble radioactivity, and reflect different states of erythropoietic output: genuine erythropoietic state at day -4, increased erythropoietic output at day +4, and decreased erythropoietic output at days 8 and 15 in response to decreased endogenous Epo production induced by the increase of RBC mass. It is probable that

Table 3. Analysis of Membrane Proteins by UT-PAGE

	N	Spectrin	Ankyrin	Band 3-Globin Area	α Globin
Normal mice					
-Epo	3	28.80 ± 0.35	7.00 ± 0.30	20.63 ± 0.31	2.67 ± 0.47
+Epo	6	$29.70 \pm 0.50^*$	6.95 ± 0.57	19.85 ± 0.73	3.68 ± 0.84
β thalassemic mice					
-Epo	3	18.00 ± 0.70	6.23 ± 0.81	28.47 ± 8.01	17.90 ± 3.38
+Epo	6	25.10 ± 7.53	7.47 ± 1.41	18.50 ± 5.50	$7.70 \pm 4.07^*$

Values shown are the percentages of total membrane proteins (excluding globin) in RBCs of normal or β thalassemic mice treated (+) or not treated (-) with rhEpo. Data shown are mean \pm 1 SD.

Abbreviation: N, number of mice studied.

*Significantly different with and without rhEpo ($P < .05$).

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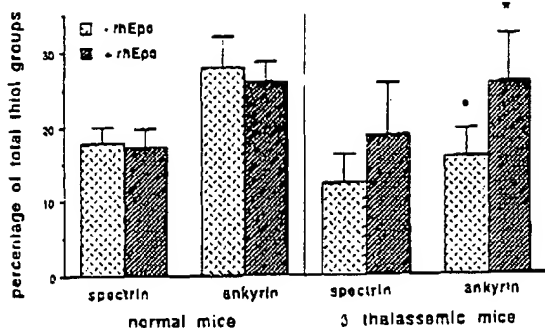


Fig 2. Thiol group reactivity of spectrin and ankyrin in normal and β thalassemic mice after treatment with rhEpo, and compared with untreated mice. Reactive thiol groups of spectrin and ankyrin are expressed as the percentage of total membrane protein thiol groups (ie, excluding globin). Data shown are mean \pm 1 SD. (●) Significantly different from the value observed in control normal mice ($P < .05$); (*) significantly different with and without rhEpo injections ($P < .05$).

rhEpo injections induced a second wave of reticulocytosis between days 8 and 15, as previously observed by Alter et al.¹⁰ We did not evaluate reticulocyte counts every day because repeated bleeding of the mice would have interfered with the effects of rhEpo injections. The slower increase in Hb with the duration of the rhEpo treatment has already been observed in normal mice,²² and could be secondary to blocking antibodies developed against human Epo, because the amino acid sequence of mouse Epo is 20% different from that of human Epo.²³ Such anti-rhEpo antibodies have been detected in the serum of W/W^m mice receiving rhEpo for 3 weeks.¹² In our study, no anti-rhEpo antibodies could be detected in any of the mice 5 days after the last rhEpo injection. The evolution of Hb under rhEpo injections could also be due to a depletion in functional iron, induced by increased erythropoiesis, as reported in humans.²⁴ However, in our study, the two normal mice that received injectable iron (2 mg/kg) on days 4, 8, and 11 had exactly the same evolution in their hematologic parameters.

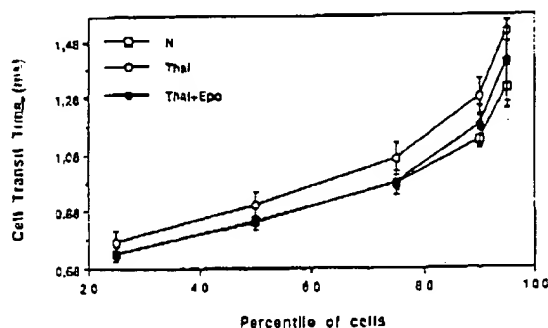


Fig 3. Deformability of normal and β thalassemic RBCs before and after rhEpo injections. Erythrocyte deformability of normal untreated mice (□) and of homozygous β thalassemic mice before (○) and after (●) rhEpo injections. The transit times required for the 25, 50, 75, 90, and 95 percentile of cells passing through the micropore filter are mean \pm 1 SD.

Nevertheless, a relative iron deficiency due to the inability of the reticuloendothelial system to release available iron cannot be completely excluded. Finally, this slower increase in Hb with the duration of rhEpo treatment could reflect the depletion of the cell pool sensitive to Epo and inducible toward final differentiation. It is possible that different treatment schedules might induce a further increase of the Hct and Hb level and overcome the limited response observed in this brief study.

The same doses of rhEpo induced a milder but nevertheless significant increase of the Hb level (+1.3 g/dL) and Hct (+4.9%) in homozygous β thalassemic mice. These results suggest that thalassemic mice are less responsive to rhEpo injections than normal mice in terms of Hb increase. Our results show that murine β thalassemia is associated with relatively high spontaneous Epo levels in the absence of rhEpo. This finding may explain the decreased response of β thalassemic mice to exogenous Epo. None of the thalassemic mice was administered iron, because of the spontaneous iron overload that has been reported in these mice.^{25,26} As in normal mice, no anti-rhEpo antibodies could be detected in any of the mice.

Increase of β minor globin chain synthesis has been shown to occur under conditions that are associated with enhanced HbF synthesis in humans. β minor chain synthesis declines during development,² being 45% of adult-type β globin chain synthesized at day 14 of gestation and reaching adult values of 23% of total β globin synthesis at day 15 after birth. A greater switch in adult-type β globin chain synthesis has been reported in mice producing a variant β single globin chain.²⁷ In these mice, the relative level of β^2 minor decreases through fetal development from 80% of adult-type β globin chains synthesized at day 11.5 of gestation to 29% at day 6 after birth. β minor Hb is elevated when the hematopoietic system is stressed by phlebotomy,⁸ in α or β thalassemia,¹⁰ or in congenital macrocytic anemia.⁹ Moreover, β minor globin chain synthesis has been shown to increase in response to 5-azacytidine¹¹ or Epo,¹⁶ known to increase HbF synthesis in humans and primates.^{12,13}

The present results show the parallelism between the β minor chain synthesis and the erythropoietic output (reticulocytes and TCA-precipitable radioactivity) both in normal and β thalassemic mice. At day 4 of rhEpo treatment, the β minor/ α ratio was maximum (0.41 in normal mice; 0.81 in β thalassemic mice). This ratio was minimum when the erythropoietic output was minimum, at day 15, four days after the last rhEpo injection (0.24 for normal mice; 0.67 for β thalassemic mice). The intermediate values in the steady state (day -4, 0.36 and 0.75, respectively) probably reflects the genuine erythropoietic response. The increase in β minor chain synthesis occurred rapidly after the first rhEpo injection. Similarly, the proportion of β minor chain synthesis was rapidly decreased after the last rhEpo injection, when the reticulocyte output was minimum. A decrease in β minor chain synthesis has been shown to occur during the differentiation of Epo-responsive cells.²⁸ Our results suggest that rhEpo modulated β minor chain synthesis during the late erythroid differentiation and maturation.

Murine β thalassemia is characterized by a relatively mild globin chain imbalance, and disease expression is mainly due to the large excess of insoluble α chains that arise from the absence of significant proteolysis and great instability of murine α chains.¹ It was thereby possible that a small reduction in globin chain imbalance could have significant effects at the cellular level and reduce significantly membrane abnormalities.

In normal mice, rhEpo had little effect on RBC membrane proteins or on their thiol group reactivity, and no significant effect on the cellular deformability. In contrast, in β thalassemic mice, rhEpo injections decreased the cellular defects by decreasing the amount of insoluble α globin, increasing the proportion of spectrin, and reducing the proportion of abnormal protein fractions between band 3 and globin. Moreover, thiol group reactivity of spectrin and ankyrin was improved after Epo injections, indicating a reduction in the oxidation of membrane proteins. These changes in the membrane structure were reflected at the functional level, because cellular deformability was normalized. These results are in agreement with the data reported by Sorensen et al.,²³ who showed that reduction in membrane-bound α globin was associated with significant changes in cellular deformability in transgenic homozygous β thalassemic mice expressing human β^S transgene at a low level (10% of total β globin chains).

In human β thalassemia intermedia, a small increase in Hct. might reduce the need for transfusions. The dramatic improvements induced by rhEpo on membrane proteins and cellular deformability suggest that the short life span of β thalassemic RBCs could possibly be improved. Several studies have shown that HbF synthesis could be increased under rhEpo injections in primates such as baboons¹⁵ and rhesus monkeys.²⁰ This increase in HbF synthesis can be potentiated by the combination of different agents such as

hydroxyurea,²¹ and hemopoietic growth factors such as interleukin-3 or granulocyte-macrophage colony-stimulating factor.²⁰ A recent study reported that Epo did not enhance HbF synthesis in sickle cell disease, and did not improve the effect of hydroxyurea.²² However, rhEpo was administered at relatively low doses (1,500 U/kg twice a day, one day a week) to avoid an increase in RBC mass and blood viscosity that could induce vasoocclusive crisis. Indeed, the doses used in this study did not even increase reticulocyte counts nor Hb level. It is likely that larger doses of rhEpo are necessary to increase HbF synthesis, and β thalassemic patients do not present similar risks of vascular occlusion when increasing their RBC mass. One might ask whether Epo therapy in β thalassemic patients would not induce further expansion of the marrow and additional bone damage. An improved erythropoiesis due to enhanced HbF synthesis and reduced globin chain imbalance, should ameliorate peripheral oxygenation, and thereby reduce the need for bone marrow expansion. Moreover, the use of cytotoxic agents such as hydroxyurea, known to increase by itself HbF synthesis in humans, in association with rhEpo, could prevent such deleterious side effects.

We conclude that homozygous β thalassemic mice are responsive to rhEpo, although to a lesser extent than normal mice. The synthesis of β minor globin chain depends on the erythropoietic stimulation and output in normal, as well as in β thalassemic mice. rhEpo increased Hb and Hct, reduced membrane abnormalities, and ameliorated the rheologic properties of β thalassemic cells. If rhEpo proves to be as effective in increasing HbF synthesis in humans as it is in primates, it could be of interest to consider its potential application in the human counterpart of murine β thalassemia, ie, human β thalassemia intermedia.

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